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1 Investigation of the enhanced antimicrobial activity of combination dry powder
2 inhaler formulations of lactoferrin

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ABSTRACT

The airways of most people with cystic fibrosis are colonized with biofilms of the Gram-negative, opportunistic pathogen *Pseudomonas aeruginosa*. Delivery of antibiotics directly to the lung in the form of aerosols or dry powders offers the potential to achieve high local concentrations directly to the biofilms. Unfortunately, current aerosolised antibiotic regimes are unable to efficiently eradicate these biofilms from the airways. We investigated the ability of the innate antimicrobial, lactoferrin, to enhance the activity of two aminoglycoside antibiotics (tobramycin and gentamicin) against biofilms of *P. aeruginosa* strain PAO1. Biofilms were prepared in 96 well polystyrene plates. Combinations of the antibiotics and various lactoferrin preparations were spray dried. The bacterial cell viability of the various spray dried combinations were determined. Iron-free lactoferrin (apo lactoferrin) induced a 3 log reduction in the killing of planktonic cell by the aminoglycoside antibiotics ($p < 0.01$) and also reduced both the formation and persistence of *P. aeruginosa* biofilms ($p < 0.01$). Combinations of lactoferrin and an aminoglycoside displays potential as an effective new therapeutic strategy in the treatment of *P. aeruginosa* biofilms infections such as those typical of the CF lungs.

KEYWORDS: Antimicrobial protein, spray drying, *Pseudomonas aeruginosa*, cystic fibrosis, biofilms, dry powder inhalers.

1. INTRODUCTION

P. aeruginosa biofilms have been recognised as one of the most persistent microbe community in the airway of people with cystic fibrosis (CF) (Singh et al., 2002; Banin et al., 2006; Costerton et al., 1999; Musk and Hergenrother, 2008; Van Delden and Iglewski, 1998). We now understand that *P. aeruginosa* are able to form these biofilms by assembling viable cells together to form a cooperative consortium encased within an exopolysaccharide (or glycocalyx) (Gomez and Prince, 2007; Lam et al., 1980; Singh et al., 2000). The exopolysaccharide formed specialises in attaching to a variety of surfaces (Mah and O'Toole, 2001). Consequently, the presence of *P. aeruginosa* biofilms at any stage of infection usually results in recurrent bacterial infections due to the ability of dispersed planktonic daughter cells released from *Pseudomonas* biofilms to colonise new surfaces (Hoiby et al., 2001). Currently, in order to manage the high incidences of mortality resulting from chronic colonization of *P. aeruginosa* in the airways, clinicians have employed the use of aerosolised antibiotics (O Riordan, 2000; Ramsey et al., 1993). Many of the established antibiotic therapies such as colistimethate sodium and tobramycin are delivered to the CF airways using nebulisers. Nebulising medication is time consuming and requires the use of expensive nebuliser apparatus that require maintenance. Both these factors increase the treatment burden and may reduce compliance and, therefore, treatment efficacy. New dry powder inhaler (DPI) formulations of colistimethate sodium (Colobreathe; Forest Laboratories, Dartford, UK) and tobramycin (TOBI Podhaler; Novartis Pharmaceuticals, Camberley, UK) have become available,

79 delivered by convenient portable mechanisms (Turbospin device (Forest
80 Laboratories) and T-326 Inhaler (Novartis Pharmaceuticals)). The benefits of
81 dry powder antibiotics are that they are quicker to use and easier to maintain
82 than traditional nebulised therapy, which may thereby improve adherence to
83 the therapy

84 Although aerosolised antibiotic therapy has its merits, such as; the ability to
85 achieve high local concentrations in the airways, decrease systemic toxicity
86 and successful suppression of established planktonic *P. aeruginosa* cells it is
87 relatively inefficient in the eradication of persistent biofilms of *P. aeruginosa*
88 (A. L. Smith et al., 1999). In fact, several studies have suggested that when
89 bacterial cells exist in biofilms, they can become 10 – 1000 times more
90 resistant to the effects of antimicrobial agents (Rogan et al., 2004; Smith,
91 2005) as well as to the effects of immune cells (Leid et al., 2002; Jesaitis et
92 al., 2003). Evidence from several studies suggests that once *P. aeruginosa*
93 biofilms are established in the airways, they become a formidable, highly
94 organized and resistant pathogen with fatal consequences for the host.
95 Hence, loss of any defence mechanism by the host that may facilitate biofilms
96 development could result in catastrophic consequences (Rogan et al., 2004).

97 The antimicrobial peptides and proteins (AMP) form an important component
98 of innate immune defences- the primary defence system of the majority of
99 living organisms. These AMP's are present in high concentrations on
100 respiratory epithelia and are able to delay or prevent microbial growth
101 following an infection. Usually, they can act for several hours until the
102 adaptive immune system is sufficiently mobilised to the site of infection. An

example of one of these AMP's is lactoferrin, a glycoprotein of about 80 kDa found in high concentration in most exocrine secretions including milk, tears, saliva, genital secretions, intestinal mucus and in the specific granule of neutrophils (Ellison, 1994). Various biological functions of lactoferrin – i.e. anti-microbial, anti-inflammatory and immunomodulatory – have been described (Tomita et al., 2002).

Lactoferrin displays antibacterial effects against planktonic Gram-negative microbes by two mechanisms; (i) impairment of bacterial multiplication by decreasing the availability of iron which is required for bacterial growth (Rogan et al., 2004; Arnold et al., 1982) and (ii) disruption the outer membrane of the microbes by binding to lipopolysaccharide (LPS) this results in an altered permeability eventually resulting in the death of the microbe (Elass-Rochard et al., 1995; Appelmek et al., 1994). More recently, lactoferrin has been shown to inhibit *P. aeruginosa* biofilm formation by sequestering iron from the microbe (Singh et al., 2002). This biofilm inhibitory property is unique to lactoferrin and is a pivotal role in host defence against invading microbes. Several studies have so far shown that lactoferrin is able to enhance the antimicrobial properties of some antibiotics (Leitch and Willcox, 1999). This is particularly interesting since currently inhaled antibiotics (for example tobramycin) has been frequently associated with emergence of resistant strains (O Riordan, 2000; Costerton et al., 1999; Allison et al., 2011). The aim of the present study was to investigate the development of combination DPI formulations of lactoferrin with other anit-microbial agents using spray drying. The further focus of our study was to investigate if these

treatment strategies permit the prevention of biofilm formation and/or disruption of persistent biofilms of *P. aeruginosa* in patients whose airways have been compromised to *P. aeruginosa* infection.

2. METHODS AND MATERIALS

2.1 Bacterial strains.

P. aeruginosa strain PAO1 was routinely grown from CAA medium- nutrient medium containing casamino acids (CAA, 0.5% w/v) supplemented with 1mM magnesium chloride. For the biofilm penetration assays, the BSAC standard *Escherichia coli* NCTC 10418 was used and was grown overnight in Luria-Bertani (LB) broth.

2.2 Production of iron-free lactoferrin (apolactoferrin; ApoLf).

Apolactoferrin was prepared by dialyzing bovine lactoferrin (kindly donated by DMV Fonterra, Netherlands) against 20 mM sodium acetate, 20 mM sodium dihydrogen phosphate and 40 mM EDTA (pH 3.5) for 24 hrs at 4°C. The apolactoferrin was dialysed against at least two changes of 900 ml distilled water and either used immediately or stored at -80°C until needed. Ferric ion (Fe^{3+}) removal was confirmed by atomic absorption spectroscopy (data not shown).

2.3 Production and characterisation of mono and combination particles of lactoferrin and apolactoferrin with Tobramycin or Gentamicin.

A spray drying technique was employed for the preparation of micron-sized particles of lactoferrin (LF), apo-lactoferrin (ApoLf) tobramycin (Tobi) and gentamicin (Genta). Aqueous solutions of each material (2% w/v) was spray-dried using a Büchi 290 Mini Spray Dryer with pneumatic atomizer with a 7mm aperture, air (prior relative humidity 20%) aspirator rate 35 m³/h, feed pump 5 mL/min, spray flow rate 700 L/h, inlet air temperature 180– 190°C, outlet temperature 96–98°C.

Inhalable combination particles of each antibiotic and LF and ApoLf were also produced by spray drying. Aqueous solutions of tobramycin and gentamycin with and with micronisation of the different formulations using a (Büchi Laboratoriums-Technik AG, Flawil, Switzerland).

For particle sizing, dry powders were dispersed with compressed air (3 bar) and sized by laser diffraction (RODOS dry powder feeder; HELOS laser diffractometer, WINDOX 4.0 software; Sympatec GmbH, Germany). The 10%, 50% and 90% undersize particle size values (X10, X50 and X90, respectively) were obtained.

The in vitro aerosolisation performance of the dry powders was measured using a Multi-stage liquid impinge (MSLI, Copley Scientific, Nottingham, UK).

The four stages of the MSLI were charged with 20 mL of water. A vacuum pump was attached to the MSLI and the flow rate was set at a flow rate of 60

L/min using a digital flowmeter (DFM2000, Copley Scientific, Nottingham, UK). Approximately 30 ± 2 mg of each of spray dried powders was manually weighed into a size 3 hard hydroxypropyl methylcellulose (HPMC) capsules (V-caps, Capsugel, Geel, Belgium). A capsule was placed into the capsule compartment of a CyclohalerTM DPI device (Pharmachemie BV, The Netherlands), pierced and actuated for 4 s. Powders depositing in the four impinger levels were recovered by agitating the apparatus to ensure complete dissolution. The powder which deposited in the throat and the back filter were also collected and the amount of protein depositing in each of these stages determined by carrying out bicinchoninic acid (BCA) assay or using a high performance liquid chromatography (HPLC) assay for the detection and quantification of either Tobi or Genta.

Quantitation of total protein was carried out according to the manufacturing instructions accompanying a BCA assay kit (Sigma Aldrich, Poole, UK). Briefly, bovine serum albumin standards were prepared by diluting the contents of a 1 mL ampoule containing 2 mg of albumin (to prepare standard concentrations of 20 – 2000 $\mu\text{g/mL}$). The working reagent was prepared mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. Then a 200 μL aliquot of the working reagent was added into each of the wells already containing 25 μL of either standard or test protein. The plates were covered and incubated at 37 °C for 30 minutes, then cooled to room temperature and then the absorbance measured at 562 nm using a Multiskan Spectrum - UV/Vis Microplate Spectrophotometer.

Tobi was separated and quantified by HPLC (Agilent 1260, London, UK) using

an isocratic mobile phase consists of buffer 0.05 M diammonium hydrogen phosphate, pH adjusted to 10.0 using tetramethyl ammonium hydroxide. Chromatography was carried out at 25°C on a Purosphere RP-8e, 250 mm × 4.6 mm, 5mm (Merck KGaA, Darmstadt, Germany). The detection was carried out using variable wavelength UV-Vis detector set at 210 nm. The compounds were eluted isocratically at a steady flow rate of 1.0 mL/min. Gentamicin was separated and quantified by HPLC (Agilent 1260, London, UK) using an isocratic mobile phase was 48.5mM TFA–MeOH (97:3, v/v) and ran at flow rate of 0.7 ml/min. Chromatography was carried out at 25°C on a Hypurity RP 18, 3µm, 125 mm x 4 mm i.d. (Thermo Hypersil, Runcorn, UK. The detection was carried out using an evaporative light scattering detector (Agilent, London, UK).

The aerosolisation performance of the different spray-dried powders were characterised by comparing the percentage emitted fraction (%EF, percentage collected from all stages of MSLI as a function of recovered dose) and fine particle fraction of the recovered dose (FPF_{RD}, percentage of FPD to RD) of the respective spray- dried powders. The %EF, FPF_{RD} were expressed as the means of quintuplicate runs (n = 5).

2.4 Viability assay for planktonic bacterial killing.

The antimicrobial activity of Lf and apoLf, alone and in combination with the aminoglycosides, towards planktonic bacteria was assessed using a viability assay. Briefly, 10µL of ~ 6.6 x 10⁷ CFU/ml PAO1 was incubated with 90 µL of

2 $\mu\text{g/ml}$ of Lf, ApoLf or combinations of protein with aminoglycoside. Samples were taken every 10 mins, diluted in deionised water and plated on Mueller-Hinton agar. Following 24 h incubation at 37°C , colonies were enumerated and the percentage of viable cells was determined relative to the cell count in the absence of proteins/antibiotics.

2.5 Qualitative analysis of biofilms.

Crystal violet staining was used to assess the effects of spray-dried formulations of Lf, ApoLf and combinations of proteins with aminoglycosides on biofilm formation by PAO1. Overnight cultures were diluted 1:100 (v/v) into fresh medium supplemented with magnesium chloride, glucose and casamino acids. In order to examine the effects of compounds on biofilm *formation*, aliquots were dispensed into a 96 well plate in the presence of the compounds at 2 g/ml . Plates were incubated at 37°C for 24 h, after which time excess broth was removed and the biofilm was stained with crystal violet. In order to examine the effects of the compounds on a pre-formed biofilm, overnight cultures, diluted 1:100 as above, were incubated at 37°C for 24 h before compounds were added and plates incubated for a further 24h prior to biofilm staining. In both cases, following the appropriate incubation times, the adherent biofilm was washed twice with distilled water before staining with 0.1% (w/v) crystal violet in distilled water for 15 mins at room temperature. Following vigorous washing with water, the crystal violet-stained biofilms were solubilised in 30% (v/v) acetic acid and the absorbance at 550nm was

determined (Multiskan spectrum plate reader) using 30% acetic acid in water as the blank.

2.6 Preparation of biofilms for penetration assays.

Biofilms of *P. aeruginosa* were prepared according to a method adapted from Anderl *et al.* (2000). Briefly, cultures of *P. aeruginosa* were grown overnight at 37°C in casamino acid media supplemented with magnesium chloride. The bacterial culture was diluted to give ca. 10⁶ cfu/ml and 5 µL of this diluted inoculum was used to seed a UV-sterilised 13 mm diameter, 0.2 µm pore size polycarbonate nucleopore membrane (Whatman). Membranes were placed on Mueller-Hinton agar (MHA) plates and incubated for 24h at 37°C after which time they were transferred onto fresh MHA for a further 24h. This established biofilm was then used in the assembly depicted in Fig. 1.

2.7 Biofilm penetration assay.

E. coli NCTC 10148 was grown overnight in Luria-Bertani (LB) broth before the density was adjusted to a 0.5 McFarland standard and applied to MHA plates to enable the growth of a confluent lawn. The biofilm penetration of tobramycin and gentamicin, either alone or in combination with the lactoferrin preparations, was determined using a method modified from Singh *et al.* (2010). Briefly, the membranes supporting biofilm growth were transferred to MHA plates inoculated with *E. coli*. A UV-sterilised 6 mm diameter, 0.2 µm

pore size nucleopore polycarbonate membrane (Whatman) was placed on top of each biofilm together with the antibiotic disc, containing the compounds of interest. All plates were incubated at 37°C for 24 h before the diameters of the clear zones of inhibition of growth were measured.

Controls comprising of membranes and antibiotic discs without biofilms and biofilms with “empty” discs were also set up. Antibiotic discs containing tobramycin, gentamicin and combinations of both antibiotics with the different preparations of lactoferrin were all prepared locally. Antibiotic discs were applied to the agar following inoculation with *E coli* and plates were incubated for 24 hours at 37°C. Zones of inhibition were measured after this incubation period and used to construct a calibration curve for antibiotic effects. A calibration plot of the diameter of the zone of inhibition against log concentration (for antibiotic discs containing 1 – 10,000 µg) was used to determine the concentration of the antibiotic in the combination discs that passed through the biofilms. The diameter of the zones of inhibition in the controls (no biofilm) were taken to signify 100% penetration and used to determine the percent penetration of the antibiotics through the biofilms. All experiments were performed in triplicates and a two-tailed, paired t-test was used for statistical analysis. In order to avoid false negative results arising from side diffusion from the antibiotic disc around the biofilm, rather than through the biofilm, only biofilms <13 mm in diameter were used in this assay.

2.8 Confocal imaging of non-viable bacteria in biofilms.

294

295 Reusable silicone incubation chambers (Flexwell™, Grace Bio-Labs) were
296 used to create 8 chambers on glass coverslips into which *P. aeruginosa* was
297 seeded and incubated for 72 hours in order to produce biofilms. A 1ml aliquot
298 of the cell suspension ($\sim 10^7$ cfu/ml) was added into each of the chambers
299 and incubated at 37°C, with CAA medium refreshed every 16 hours. Following
300 72 hours growth of the biofilms on the coverslip, the CAA medium was
301 removed and wells were carefully washed twice with phosphate-buffered
302 saline (PBS) to remove planktonic bacteria. Biofilms were treated for 24 hours
303 at 37°C with 200 μ l of 1 g/ml tobramycin, ApoLf or Tobi+ApoLf. Positive
304 controls (viable bacteria) contained 200 μ L of CAA media without any
305 antibiotic, whilst the negative control (dead bacteria) was treated with 200 μ L
306 of isopropanol. The biofilms were washed with PBS and stained with
307 LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, Life Technologies,
308 Ltd, Paisley, UK) using a mixture of 3 μ l of SYTO® 9 with 3 μ l of propidium
309 iodide per ml of saline, as per the manufacturer's instructions.

310 Visualisation of antibiotic/combination treated and untreated biofilms was
311 carried out using a Leica tandem confocal scanner SP5 confocal laser
312 scanning microscope (CLSM; Leica Microsystems, Milton Keynes, UK).
313 Images were obtained using 20x HC PL APO with 20x/0.70 CS lens. For
314 detection of SYTO® 9 (green channel), the 488 nm line of the argon laser with
315 a detection bandwidth of 495 – 515 nm was employed. For detection of
316 propidium iodide (red channel), the 561 nm HeNe laser was used with a
317 detection bandwidth of 615 – 600 nm. Image analysis was carried out using

318 Leica LAS AF 2.2.1 software.

319

320 2.9 Statistical analysis.

321

322 All experiments were performed in independent triplicate analysis of multiple

323 repeats. Two-tailed, unpaired t-tests or two-way Anova with Tukey's post hoc

324 were used for statistical analysis. Statistical analyses were performed using

325 GraphPad Prism 5.

3. RESULTS

The effect of spray drying on the antimicrobial activity of Lf and ApoLf was evaluated for planktonic *P. aeruginosa* PAO1 over a 60-minute incubation period (Fig. 2). Lf produced over 2 log reductions in bacterial number after 60 minutes, whereas SD Lf was slightly less effective (<2 log reductions in 60 minutes). The kinetics of the action of Lf and SDLf were very similar, with most activity apparent in the first 10 minutes of incubation. ApoLf was more effective than Lf - 60 minutes incubation with ApoLf reduced bacterial numbers by over 5 log₁₀ cfu/ml. Although spray drying reduced the activity of ApoLf, this was still greater than Lf - indicating that ApoLf, even when spray dried, is the more active antimicrobial form of the protein.

Aminoglycosides were spray dried with the antimicrobial proteins and tested for their ability to kill planktonic organisms. The data in Fig. 4A indicated that spray dried tobramycin at 2µg/ml produced a 2 log reduction in cfu/ml over 60 minutes, but that this activity was significantly ($p<0.05$) increased when the antibiotic was co-spray dried with either Lf or ApoLf. The combination of TOBI with ApoLf was the most effective and resulted in over 5 log reductions in cfu over duration of the assay. The combination of Genta with Lf or ApoLf was also more effective in terms of planktonic bacterial killing in comparison to the mono-treatment with the aminoglycoside alone (Fig. 4B). Here, spray dried Genta was shown to be effective and reduce the cfu by 2 logs over 60 minutes, but when Gm was co-spray dried with Lf, this killing was enhanced to

almost 4 log reductions and again, ApoLf with Gm was most effective and resulted in 100% bacterial eradication within 40 minutes.

The particle size distribution (PSD) and aerodynamic performance of the spray dried and co-spray dried formulations is shown in Table 1A and B. The d_{50} of the spray dried and co-spray dried particles was between 1.5 – 1.9 μm with 90% of particles below 5 μm .

Following aerosolisation of the different spray dried formulations, the %EF ranged between 88 – 97%. Whilst %EF is a measure of how well the powder is removed from the device, the FPF_{RD} is the measure of the aerosolisation efficiency. The %FPF of the spray dried Lf and ApoLf was lower than that of Tobi and Genta. The co-spray dried formulation also had high %EF (range: 91 – 94% for both agents) and the %FPF ranged from 35 – 42% for both the antibiotic and protein fraction of the co-spray dried particles. Moreover, the %FPF of each agent was very similar suggesting that were likely to be co-delivered.

The effects of the spray dried antibiotics and combinations were examined for their effects on biofilms of PAO1 using the crystal violet assay. Figure 5A shows that SD TOBI reduced the biofilm intensity in the first 24 hours to 57.4 \pm 0.1 % control. However, co-spray drying TOBI with either Lf or ApoLf significantly ($P<0.01$) enhanced this reduction to 24.8 \pm 0.8 or 22.9 \pm 0.8 % control, respectively. A similar pattern of biofilm inhibition was observed after

24-hour treatment with SD Genta, which suggested that biofilm intensity was reduced to $56.9 \pm 7.8\%$. Co-spray drying Genta with Lf or ApoLf showed significant ($P < 0.01$) reduction in biofilm intensity to 30.5 ± 1.5 or $23 \pm 0.6\%$ control following co-spray drying Genta with Lf or ApoLf, respectively. Interestingly, the combination treatments appeared more effective on pre-formed biofilms (Fig. 5B). Here, biofilms were allowed to form for 24 hours prior to the addition of SD aminoglycosides or co-spray-dried protein-antibiotic formulations. Under these conditions, the effects of Lf-aminoglycoside combinations were less impressive, with SD TOBI+Lf reducing the biofilm intensity to $63.7 \pm 11.6\%$ whilst co-spray dried Genta+Lf only reduced intensity to $86.7 \pm 10.3\%$. However, spray-dried combinations of the aminoglycosides with ApoLf were still effective at significantly ($P < 0.01$) reducing biofilm intensity to 27.4 ± 7 or $31.5 \pm 7.8\%$ for co-spray dried TOBI+ApoLF or Genta+ApoLf respectively.

Since the SD combinations were effective at reducing biofilm intensity, the mechanism of action of this effect was investigated by examining the effects of the antimicrobial proteins on penetration of the SD antibiotics through PAO1 biofilms. The zones of growth inhibition of the indicator organism in the absence of a biofilm were taken to represent 100% penetration and used to determine the percentage retardation of the penetration of antibiotics and combinations through PAO1 biofilms. The limit of detection was taken to be 13mm, since this was size of the polycarbonate membrane filter on which the biofilms were grown.

398

399 Figure 6 indicated that PAO1 biofilms reduced the penetration of SD TOBI to
400 $15.3 \pm 6.3\%$ and spray dried combinations of Lf with TOBI did not significantly
401 alter this (drug penetration $23 \pm 4\%$ control). In contrast, the spray-dried
402 combination of ApoLf with TOBI significantly ($P < 0.05$) increased penetration
403 of the antibiotic through PAO1 biofilms to $42.4 \pm 11.7\%$. Visualisation of viable
404 bacteria within the biofilm (Fig. 7) indicated that SD combinations of ApoLf
405 with TOBI are more effective at reducing bacterial viability than SD TOBI
406 alone. Biofilms were grown on glass slides for 72 hours in order to allow
407 confocal fluorescence microscopy using the nucleic acid stains, SYTO 9 and
408 propidium iodide. While SYTO 9 stains all nuclei acid in a population (and
409 therefore bacteria with intact and damaged membranes appear green),
410 propidium iodide stains only bacteria with damaged membranes. When the
411 two dyes are used together, propidium iodide staining of damage bacteria will
412 cause a reduction in their green fluorescence, visible as an increase in the
413 yellow intensity seen on overlay of the green and red channel images. These
414 experiments evaluated the effects of co-spray dried TOBI+ApoLf, since this
415 combination consistently performed most effectively. Fig. 7B shows that SD
416 ApoLf reduces bacterial viability (apparent from the yellow staining in the right
417 panel), whereas SD TOBI is not altering bacterial viability and the staining is
418 predominantly in the green channel (Fig. 7C). The images in Fig. 7D
419 indicated that co-spray dried TOBI+ApoLf was most effective in terms of
420 reducing bacterial viability in the biofilm- thus red fluorescence was greater
421 here than with the other compounds tested and the intensity of the yellow

overlay indicating dead bacteria is greatest after incubation with co-spray dried TOBI+ApoLf.

4. DISCUSSION

The idea of combining various antibiotics to increase their efficacy has been around for decades. In the present study, we investigated the in vitro antibacterial activities of a novel inhaled antibiotic combination consisting of a 1:1 spray dried combination of aminoglycosides and an antimicrobial peptide (Lactoferrin). Upon spray drying our various preparations, we obtained an average residual moisture content of between 3.2 – 5.9 % indicating that the drying process was efficient in evaporating most of the moisture.

To better understand the activities of our paired agents against both planktonic and biofilms of *P.aeruginosa*, we carried out various time-kill experiments to ascertain the rate and degree of bacterial killing by the individual agents as well as in combination (Fig. 2 and 4). Our results, indicate that following spray drying all the antibiotics were able to reduce the colony forming unit per millilitre (cfu/ml) by 3 Log units within 60 mins when administered at high concentrations, but on combining both agents, we discovered that there was a much rapid bactericidal effect at very low concentrations compared to the individual administrations.

Similarly, in order to ascertain the effectiveness of our spray dried

combinations either in preventing biofilm formation or in depleting already formed biofilms. We employed a method which effectively measured biofilm mass by quantifying the amount of crystal violet retained by a microtitre plate in which biofilms had been previously grown, washed and stained. Our findings from these experiments, showed that there was a significant decrease in both the initiation and persistence of biofilms when incubated with the various lactoferrin preparations. Interestingly, this observed decrease in both initiation and persistence were both absent on exposing the biofilms to the aminoglycoside alone but reappears on combining the aminoglycosides with lactoferrin.

Furthermore, to better understand how our paired agents are able to disrupt the biofilms matrix, we employed the method whose schematic is briefly described in figure 1. Results obtained from our penetration experiments indicated that our tobramycin:lactoferrin combination permitted a significantly higher amount of solute through the model biofilms. However, it is understood that in vitro antibiotic activity may not always correlate with in vivo activity, particularly in CF because CF sputum has been shown to inhibit the activity of aminoglycosides (Mendelman et al., 1985; Hunt et al., 1995).

Previous studies have demonstrated that lactoferrin, tobramycin and gentamicin are able to impart negatively on the viability of *P. aeruginosa* biofilms (P. K. Singh et al., 2002; Tré-Hardy et al., 2008; Khan et al., 2011). However, the effect of micron sized combinations of lactoferrin-tobramycin

and lactoferrin-gentamicin has not been investigated. Thus, these studies show the potential benefit such a formulation would have.

Images obtained by confocal laser scanning microscopy CLSM show that both apo lactoferrin and tobramycin caused minimal cell membrane damage to majority of the biofilm cells. However, treatment of the biofilms with a 1 mg/ml spray dried combination of lactoferrin and tobramycin caused significant membrane damage to all cells indicating cell lysis in the *P. aeruginosa* PAO1 biofilm. These observations were consistent with our biofilm and penetration assays. Taken together, our confocal microscopy results indicate that on treating the biofilms with a spray dried combination therapy of lactoferrin and tobramycin there is a complete suppression of metabolic activity of biofilms due to the enhanced antibiotic penetration through disrupted cell membranes.

Overall, results from our study show that the spray dried combinations of aminoglycosides and lactoferrin appear to have a superior antimicrobial activity in *P. aeruginosa* PAO1 biofilms compared to monotherapy. Tobramycin:lactoferrin combination could be a potentially new therapeutic agent for biofilm associated infections. Also the fact that as a combination they can be homogeneously micronized means that they could potentially be employed in the management of chronic airway infections as seen amongst people with cystic fibrosis. The clinical applicability of a tobramycin:lactoferrin combination warrants future in vivo studies

Drug resistant bacterial infections are becoming more widespread with new drug approvals for inhaled antibiotics constantly declining year on year. The rise in drug resistance especially due to the ability of bacteria to exist in biofilms has limited the repertoire of effective antimicrobials with considerable potency against infections as commonly seen amongst people with cystic fibrosis. The potential for employing a combination therapy of lactoferrin and aminoglycoside as described here provides a new and large playing field for proper utilisation of our currently available antibiotic resources in the provision of therapeutic solutions to some of the current bacterial menace. It will be interesting to translate most of our understanding into clinical treatment so as to effectively fight resistant *P. aeruginosa* infections.

5. CONCLUSIONS

In conclusion, we have developed combination therapies comprising an antimicrobial protein (lactoferrin) and an aminoglycoside antibiotic (tobramycin or gentamicin), which are more effective than monotherapy at reducing planktonic cell viability and preventing biofilm formation. We have shown that these combinations have parameters compatible with delivery to the airways and suggest, therefore, that they represent an important and exciting new therapeutic strategy for treating biofilm-forming infections such as those typical of the CF lungs.

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614

615 TABLES

616

617 Table 1A. Particle size distribution of the spray-dried particles and their
 618 aerodynamic performance as determined by the percentage emitted fraction
 619 (%EF) and percentage fine particle fraction of recovered dose (%FPF_{RD}).

620

Formulation	$d[v,10]$ (μm)	$d[v,50]$ (μm)	$d[v,90]$ (μm)	%EF	%FPF _{RD}
SD Lf	0.8 ± 0.2	1.9 ± 0.3	4.08 ± 0.4	96.2 ± 0.4	28.6 ± 0.3
SD ApoLf	0.7 ± 0.3	1.7 ± 0.2	4.01 ± 0.5	93.9 ± 0.5	31.9 ± 0.6
SD Tobi	0.8 ± 0.6	1.7 ± 0.6	4.78 ± 0.8	88.9 ± 3.2	38.7 ± 1.4
SD Genta	0.8 ± 0.1	1.8 ± 0.2	4.38 ± 0.2	90.5 ± 0.1	40.7 ± 1.7

621

622

623 Table 1B. Particle size distribution of the co-spray dried particles and their
 624 aerodynamic performance as determined by the percentage emitted fraction
 625 (%EF) and percentage fine particle fraction of recovered dose (%FPF_{RD}).

626

Formula tion	$d[v,10]$ (μm)	$d[v,50]$ (μm)	$d[v,90]$ (μm)	%EF (Aminog lycoside)	%EF (Protein)	%FPF _{RD} (Aminog lycoside)	%FPF _{RD} (Protein)
SDLf + Tobi	0.9 ± 0.4	1.8 ± 0.6	4.56 ± 0.8	92.6 ± 0.3	91.9 ± 0.4	35.3 ± 2.2	36.8 ± 1.8
SD	0.9 ± 0.4	1.8 ± 0.6	4.01 ± 0.8	91.7 ± 0.3	90.9 ± 0.4	38.9 ± 2.2	37.2 ± 1.8

ApoLf + Tobi	0.3	0.3	0.3	0.8	0.9	3.9	1.5
SD Lf + Genta	0.7 ± 0.4	1.6 ± 0.5	4.05 ± 0.1	91.6 ± 0.5	92.3 ± 0.7	42.3 ± 2.3	40.9 ± 1.9
SD ApoLf + Genta	0.7 ± 0.4	1.5 ± 0.2	4.28 ± 0.3	93.5 ± 0.8	94.2 ± 0.6	35.1 ± 2.8	36.8 ± 1.2

FIGURES

Figure 1. Schematic depiction of the experimental setup used to analyse the penetration of antibiotics and combinations of antibiotics with lactoferrin/apo-lactoferrin, through *P. aeruginosa* biofilms. Biofilms were grown for 48 hours before use in this penetration assay.

Figure 2. Spray drying antimicrobial proteins antimicrobial activity. Apolactoferrin and SD apolactoferrin were significantly more effective than the iron-rich form ($P < 0.05$) and resulted in 4-5 log reductions in bacterial numbers over 60 minutes. Control (no antimicrobial protein), ■; Lactoferrin, ●; SD lactoferrin, ○; Apolactoferrin, ▲; SD apolactoferrin, △. Data are presented as mean ± SD, n=3 independent experiments.

Figure 3. Effects of the compounds on initiation of the biofilm, where compounds were present throughout 24 hours of culture (A) and the effects of

the compounds on an established biofilm, here compounds were added 24 hours after biofilm formation and incubated for a further 24 hours (B). Data are presented as a percentage of control- where the intensity of the biofilm in the absence of any agents (control) was given the value 100 % and all other data are expressed relative to this. Data are shown as mean \pm standard deviation of 8 repeats from triplicate analysis.

Figure 4. Effect of spray dried combinations of aminoglycosides with antimicrobial proteins on planktonic bacterial killing. A: Combinations of tobramycin with Lf or ApoLf and B: Combinations of gentamicin with Lf and ApoLf.

Figure 5. Effects of the combinations of tobramycin and on initiation of the biofilm, where the antibiotic or combination was present throughout 24 hours of culture (A) and effects of the combinations on an established biofilm (B).

Figure 6. Investigation of the penetration of combination therapies of antibiotic with lactoferrin or apolactoferrin PAO1 biofilms.

Figure 7. Confocal imaging of biofilms following exposure to spray-dried combinations of Tobo and ApoLf.

Figure 1

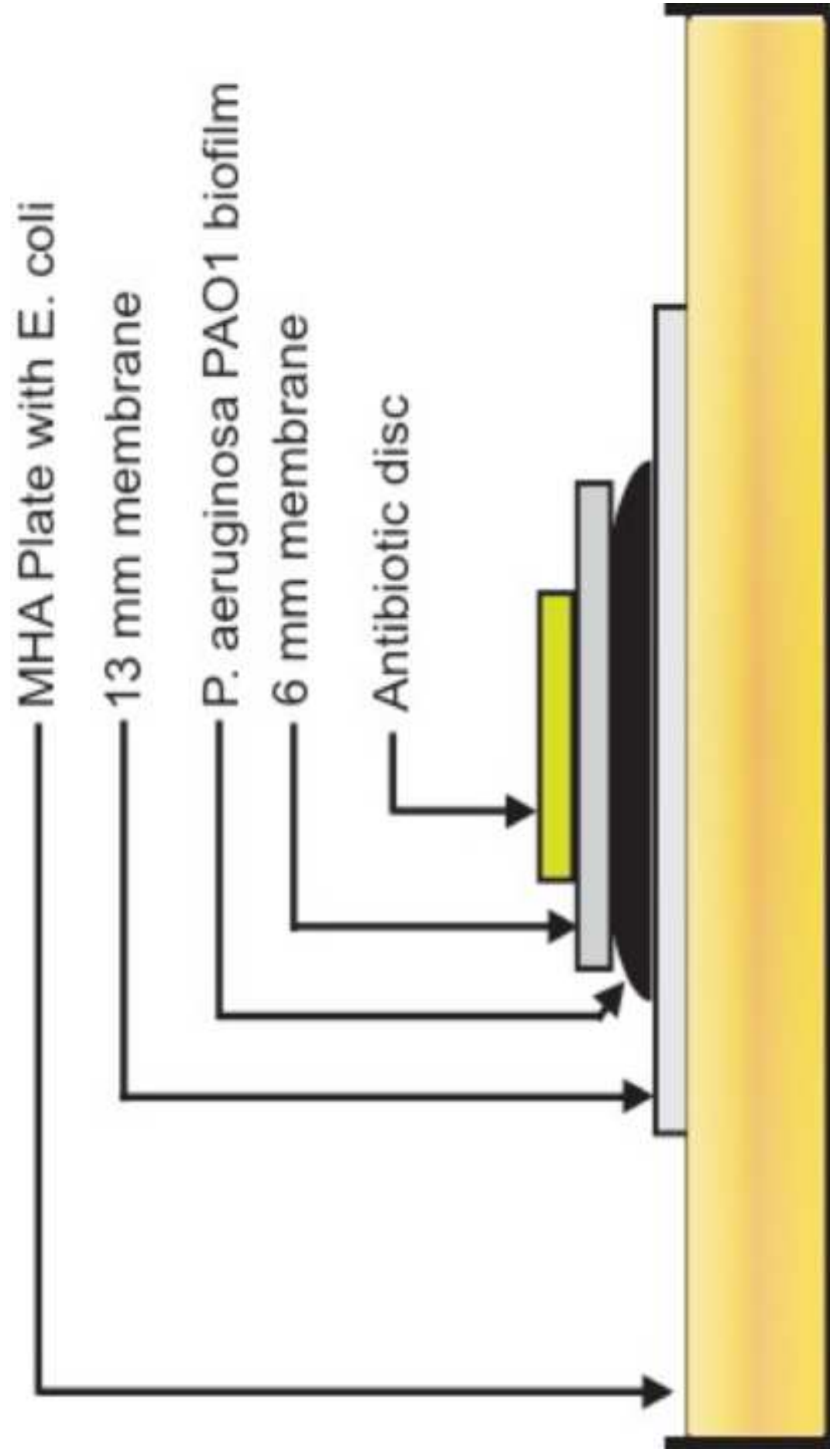
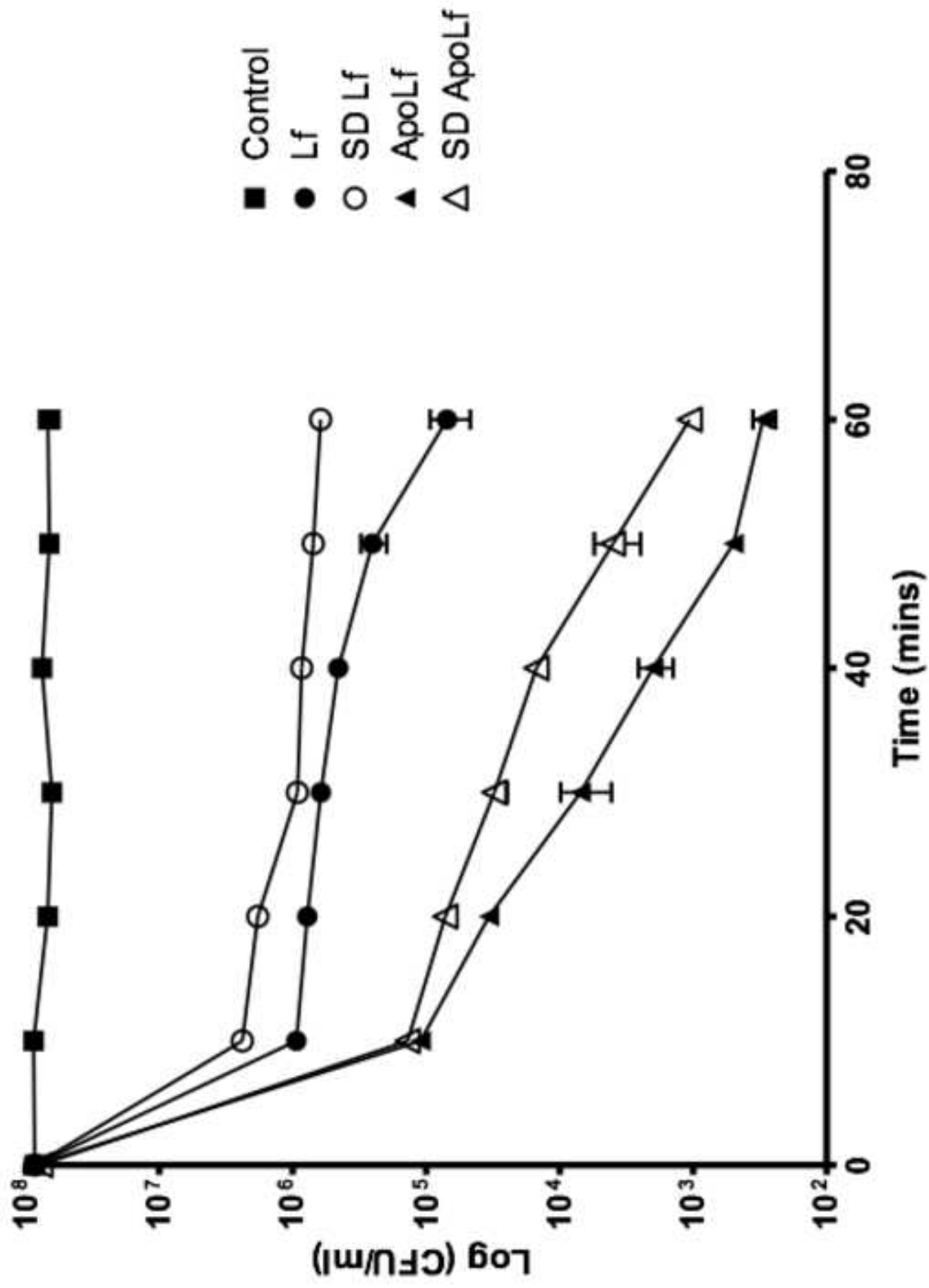


Figure 2



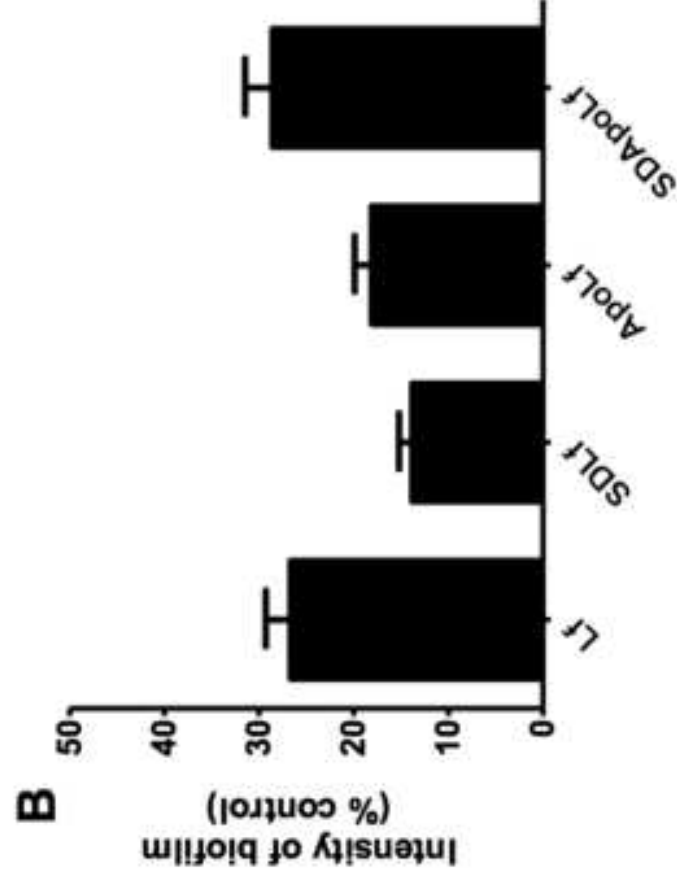
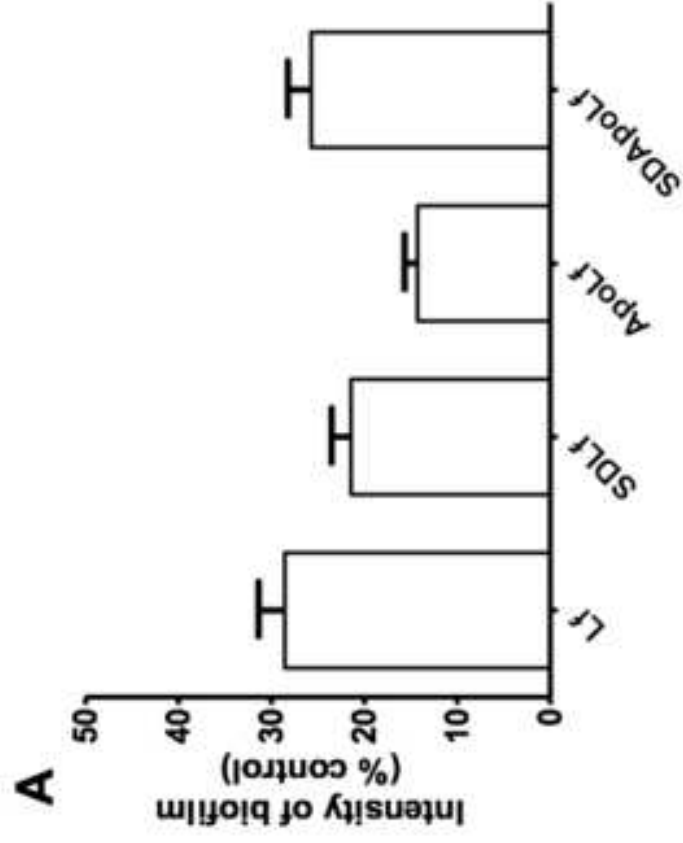
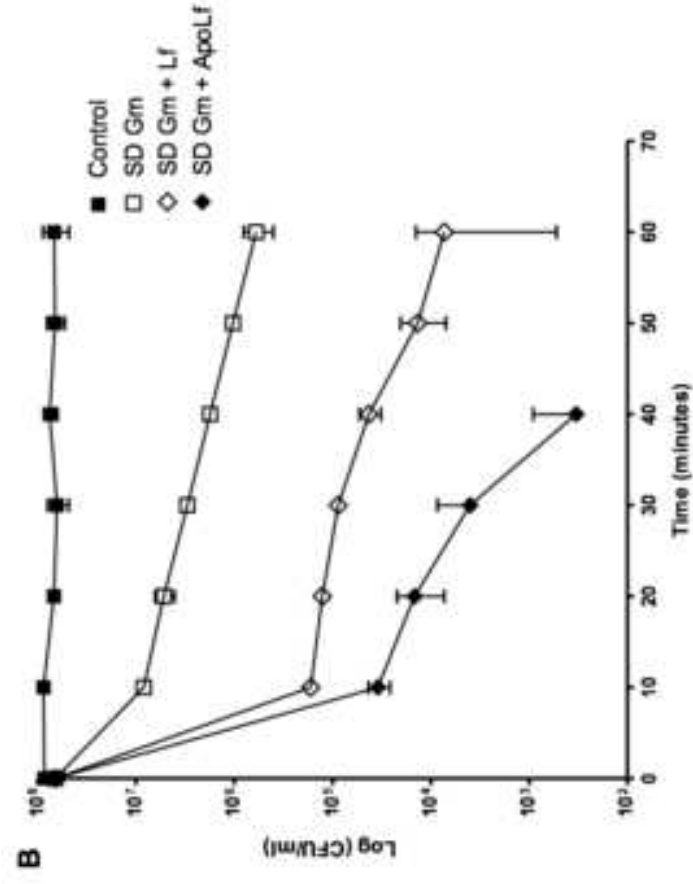
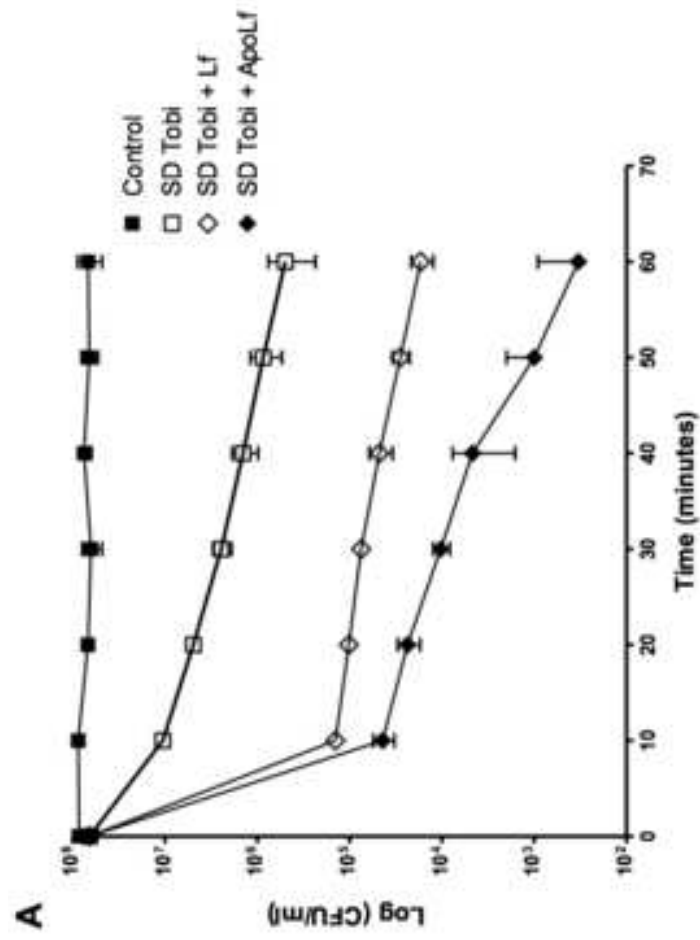


Figure 3

Figure 4



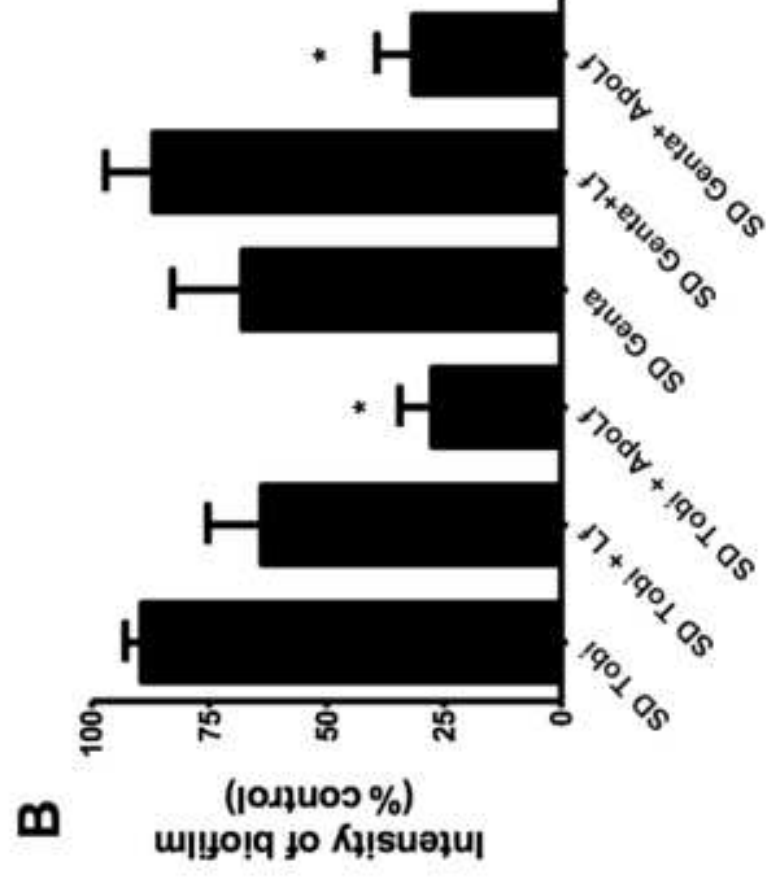
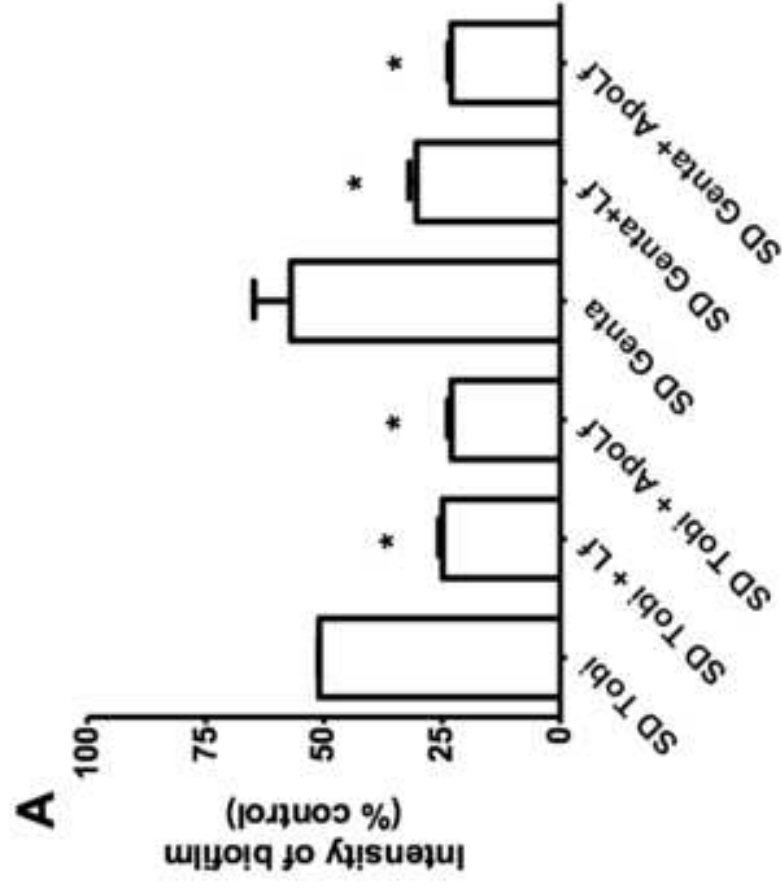


Figure 5

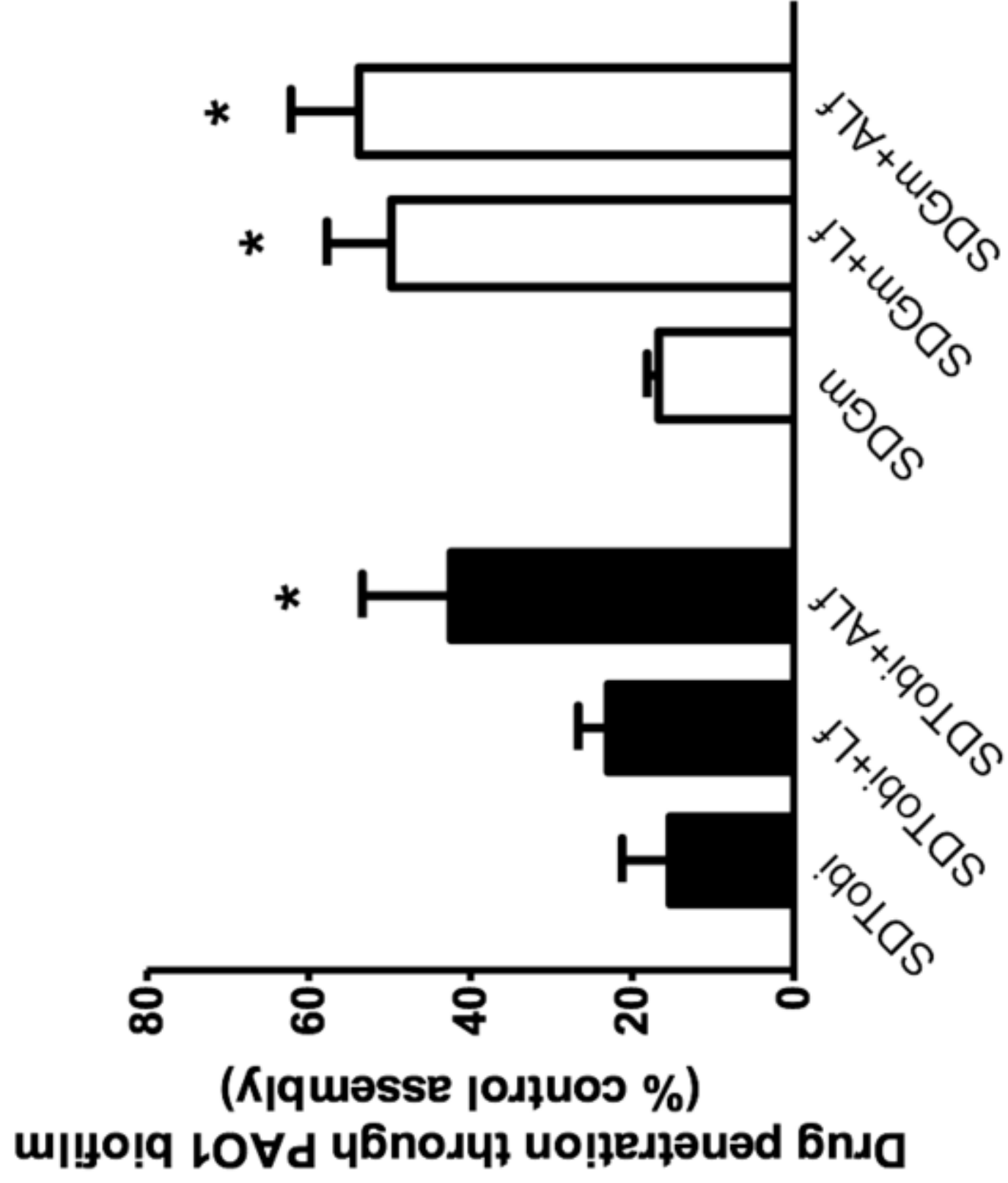


Figure 6

Figure 7

